Green tea and epigallocatechin-3-gallate are bactericidal against *Bacillus anthracis*

Shane D. Falcinelli[a*], Maggie C. Shi[a], Arthur M. Friedlander[a,b], and Jennifer Chua#[a]

Bacteriology Division, United States Army Medical Research Institute of Infectious Diseases, Frederick, Maryland, USA[a]; Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA[b].

Running Head**:** Green tea and EGCG kill *Bacillus anthracis*

#Address correspondence to Jennifer Chua, USAMRIID, 1425 Porter St., Frederick, MD 21702; Phone # 301-619-7875; Fax# 301-619-2152; Jennifer.Chua.ctr@mail.mil;

*Present address: Shane Falcinelli, University of North Carolina, Chapel Hill, North Carolina, USA.

ABSTRACT

Bacillus anthracis, the etiological agent of anthrax, is listed as a Category A biothreat agent by the United States Centers for Disease Control and Prevention. The virulence of the organism is due to expression of two exotoxins and capsule, which interfere with host cellular signaling, alter host water homeostasis, and inhibit phagocytosis of the pathogen, respectively. Concerns regarding the past and possible future use of *B. anthracis* as a bioterrorism agent have resulted in an impetus to develop more effective protective measures and therapeutics. In this study, green tea was found to inhibit the *in vitro* growth of *B. anthracis*. Epigallocatechin-3-gallate (EGCG), a compound found abundantly in green tea, was shown to be responsible for this activity, against both the attenuated *B. anthracis* ANR and the virulent, encapsulated strain *B. anthracis* Ames strain. This study highlights the antimicrobial activity of green tea and EGCG against anthrax and suggests the need for further investigation of EGCG as a therapeutic candidate against *B. anthracis*.

INTRODUCTION

Bacillus anthracis is a Gram-positive pathogen that is the etiological agent of the disease anthrax. The virulence of *B. anthracis* is attributed to three major factors: a γ-linked poly-D-glutamic acid capsule and two binary toxins, Lethal Toxin (LT) and Edema Toxin (ET). The capsule, encoded on plasmid pXO2, is believed to enhance virulence by its anti-phagocytic properties (1). The plasmid pXO1 encodes the two A-B toxins; the toxins share a binding partner, protective antigen, that pairs with either lethal factor to form LT or with edema factor to form ET. LT cleaves mitogen-activated protein kinases and ET functions as an adenylate cyclase to increase intracellular cAMP levels (2). These activities result in the dysfunction of key signaling networks in myeloid cells leading to impairment of the host innate and adaptive immune systems, and in late stages, damages to the cardiovascular system and liver (2). Human infection occurs because of exposure to *B. anthracis* spores. Infection is acquired through gastrointestinal, cutaneous or inhalational routes. Most natural infections occur through the cutaneous route with minimal mortality, whereas inhalational anthrax is much more likely to result in death (3). The prolonged environmental persistence of the spores combined with the potential for their large scale dissemination, realized in the 2001 mailing of the Anthrax Letters, has garnered the interest of the biomedical community and the public to improve current prevention and therapeutic strategies against *B. anthracis* (3).

After water, tea is the most consumed beverage in the world. Although containing little caloric value, teas have potent anti-mutagenic, anti-diabetic, anti-angiogenic, anti-inflammatory activity (4, 5). Green and black teas are dried leaves of *Camellia sinensi.* After harvest, green tea undergoes a heating process that inactivates the enzyme polyphenol oxidase. In contrast, black tea is not heated, thus allowing the enzyme to function during tea processing. Thus, both green

and black teas contain similar amounts, but different compositions of simple polyphenols (4). Whereas the polyphenol catechins predominate and represent 10 to 15% of the dry weight in green tea, these simple polyphenols have been converted to their oxidized counterparts in black tea (6). Importantly, of the four major catechins in green tea, EGCG predominates, representing more than 50% of the total catechins (7).

Green tea has potent anti-microbial activity, which is attributed to EGCG because of its relatively high abundance (8-10). EGCG demonstrates a broad range of anti-microbial activity, with inhibitory effects on viral, bacterial and fungal pathogens (11). EGCG is especially effective against Gram-positive bacteria (6, 12). The main mechanism of EGCG's anti-microbial action has been postulated to be lipid bilayer damage (13). More recent evidence suggests that EGCG also binds bacterial peptidoglycan resulting in compromise of the Gram-positive cell wall (6). In addition, EGCG has been shown to reduce survival of *Listeria monocytogenes*, *Mycobacterium tuberculosis* and *Legionella pneumophila* in macrophages by modulating cellular functions (14-16). In the context of *B. anthracis*, EGCG inhibits LT function and protects macrophages and rats from LT-induced death (17). However, to date, no data exist regarding the effect of green tea or EGCG on the growth and viability of *B. anthracis*.

First, the effect of green tea was evaluated against an unencapsulated strain of *B. anthracis* (ANR) under *in vitro* growth conditions. Next, we assessed the inhibitory activity of EGCG against *B. anthracis* ANR in human blood. Last, the promising inhibitory and killing activity against the unencapsulated ANR strain prompted us to test the activity of EGCG against the virulent, encapsulated *B. anthracis* Ames strain.

MATERIALS & METHODS

Bacterial strains and culture. Vegetative unencapsulated *B. anthracis* ANR (pXO1+, pXO2-) or encapsulated *B. anthracis* Ames (pXO1+, pXO2+) (U.S. Army Medical Research Institute of Infectious Diseases) were grown overnight from spores in liquid cultures and used to inoculate growth medium. All cultures were shaken at 37°C with 5% CO_2. All work with *B. anthracis* Ames was performed under Biosafety Level 3 containment conditions.

Growth medium preparation and blood collection. The standard growth media used were Luria Broth (LB) (Sigma, St. Louis, MO) or BBL Bovine Heart Infusion Broth (Becton Dickinson, Sparks, MD) supplemented with 40% Fetal Bovine Serum (Hyclone, Logan, UT) and 0.8% $NaHCO_3$ (BHI/FBS). In some experiments, FBS was substituted with Bovine Serum Albumin Fraction V (BSA) (final concentration 12 mg mL^{-1}) (Sigma, St. Louis, MO). Broth was combined with tea, while maintaining a constant concentration of LB, BHI and/or FBS, and inoculated 1:100 with an overnight culture of bacilli.

Whole blood samples were collected from healthy unvaccinated volunteers in vacutainers containing sodium polyanetholesulfate (BD, Franklin Lakes, NJ). Research involving human subjects was conducted in compliance with Department of Defense, Federal, and State statutes and regulations relating to the protection of human subjects and adheres to the principles identified in the Belmont Report (1979). All data and human subject research were gathered and conducted under human use protocol # FY10-09. 5 mL of normal saline (0.9% NaCl) (control) or saline containing EGCG (experimental) was added to 15 mL of whole blood for each condition.

Tea and EGCG preparation. Three tea bags (STASH © premium green or English black breakfast tea, Portland, OR) were steeped in 120 mL boiling water for 3 min and sterilized through a 0.2 µm filter. Epigallocatechin-3-gallate (EGCG) (Sigma, St. Louis, MO) was

solubilized in normal saline. Solutions of EGCG were prepared immediately prior to experiments and sterilized through a 0.2 µm filter.

Optical density (OD) measurements and Colony Forming Units (CFU) Counts. OD readings were taken at 600 nm using a Beckman DU530 Life Science UV/vis Spectrophotometer. Cultures were serially diluted and plated either on Tryptic Soy or Sheep Blood Agar Plates (Remel, Lenexa, KS) and incubated overnight at 37°C.

Live/Dead Assay and confocal microscopy. LIVE/DEAD *Bac*light Bacterial Viability Kit (Life Technologies, Grand Island, NY) was used in accordance with the manufacturer's protocol. Images were taken on the Zeiss 700 Laser Scanning Confocal Microscope System using either a 40x/1.3 or a 100x/1.4 numerical aperture oil objectives lens with the pinhole set to 1 Airy unit. All images shown are maximum intensity projections and bars on images denote 20 µm in length.

Statistical Analyses. Microsoft Excel was used to analyze data and generate graphs. Statistical significance was determined using an unpaired student's *t* - test at graphpad.com. Error bars denote standard error of the mean (SEM).

RESULTS

***Bacillus anthracis* ANR is killed in the presence of green tea.** In LB with 10% green tea, *B. anthracis* ANR growth was significantly inhibited after 4 h, with a 2 log difference between 10% green tea and the culture grown without tea (Fig. 1a). Black tea also inhibited *in vitro* growth ($p = 0.08$) but to a lesser extent than green tea (Fig. 1a). Because green tea strongly inhibited growth, the effects of time and increasing green tea concentration ranging from 10% to 50% were examined. Bacterial cultures subjected to green tea did not increase in turbidity,

whereas bacilli grown without tea did (Fig. 1b). Hence, green tea inhibits the growth of *B. anthracis* ANR.

B. anthracis forms chains of bacilli (18). To assess if green tea resulted in killing of individual bacillus within a chain, we performed a Live/Dead bacterial viability assay with confocal microscopy imaging. Bacilli in chains were manually scored live or dead based on the exclusion or inclusion of propidium iodide, respectively. Empty spaces within the chains, which are cell wall remnants of dead bacilli, were also scored as killed. In the presence of green tea, a decrease in the percentage of live bacilli was observed compared to the culture without tea (Fig. 1c & d). Interestingly, the effect of green tea on individual bacilli appeared to be random as live and dead bacilli can both be seen on a single chain (Fig. 1d). Also, killing was concentration dependent as increasing concentration of tea resulted in less live bacilli (Fig. 1c & d). Thus, green tea inhibits the growth of *B. anthracis* ANR by a bactericidal mechanism.

The presence of serum decreases the bactericidal activity of green tea. We next examined the activity of green tea in LB or BHI media that contained $NaHCO_3$ and FBS (LB/FBS or BHI/FBS). Interestingly, we found a higher percentage of live bacilli were present in LB/FBS + 50% tea versus LB + 50% tea without FBS (Fig. 2a). Similarly, a higher percentage of live bacilli were scored in BHI/FBS with tea compared to BHI with tea (Fig. 2a). This suggests that although green tea continues to inhibit bacilli growth in the presence of serum, FBS contains compounds that interfere with green tea’s bactericidal activity.

Albumin is partially responsible for interfering with the bactericidal activity of green tea. Albumin is the most abundant protein found in serum and binds a variety of endogenous and exogenous substances (19-21). To determine if albumin was responsible for

interfering with green tea's bactericidal effect, bovine serum albumin was substituted in place of FBS (BHI/BSA). Compared to BHI alone, there were more live bacilli counted after growth in BHI/BSA (Fig. 2b & c). However, there were less live bacilli in BHI/BSA than in BHI/FBS (Fig. 2b & c). Thus, BSA interferes with green tea's bactericidal effect. However, because BSA cannot recapitulate the mitigating effects of FBS fully, it also suggests that there may be other components in serum that can interfere with the anti-microbial activity of green tea. Another possibility is that the addition of BSA or FBS provides *B. anthracis* with a richer medium for growth thus increasing *B. anthracis* viability, even in the presence of green tea.

***B. anthracis* ANR killing in BHI/FBS and blood by EGCG.** Because green tea's antimicrobial activity is mainly attributed to the most abundant catechin EGCG, we next explored the effect of EGCG against unencapsulated *B. anthracis* ANR. The average EGCG concentration in brewed green tea is approximately 1-2 mM, but a range of 50µM to 4.5mM has been reported (22-24). The maximum plasma concentration of EGCG that has been observed following oral ingestion in humans is approximately 7 µM, but varies greatly (0.1 to 7 µM) and is directly proportional to the amount of tea or extract ingested (25-27). Therefore, we tested the effects of EGCG, ranging from 7 µM to 1.6 mM, on the growth of *B. anthracis* ANR in BHI broth with and without FBS. Without FBS, there was significant inhibitory activity at concentrations of 70 µM and greater (Fig 3a). Without FBS, 7 µM EGCG also inhibited growth, albeit without statistical significance ($p = 0.09$) (Fig. 3a). In the presence of FBS, there was significant inhibitory activity with 1.6 mM EGCG (Fig. 3b). 700 µM EGCG showed some growth inhibition but this did not reach statistical significance ($p = 0.09$) (Fig. 3b). When assessed by the Live/Dead assay, treatment with 1.6 mM EGCG showed significant killing of

bacilli at both 4 and 7 h in the absence of FBS. With FBS, the killing activity of EGCG was mitigated at both time points (Fig. 3c).

EGCG reaches systemic circulation after green tea consumption (25). Therefore, the effect of EGCG on *B. anthracis* ANR growth was also examined in human blood. In the presence of 1.6 mM EGCG, a growth inhibition trend was observed in the blood at 6 and 24 h, but this did not reach statistical significance (Fig. 3d). The difference in EGCG potency in media with bovine (BHI/FBS) or human serum (blood) might be explained by the differences in serum amounts or composition. Taken together, these data suggest that components in serum interfere with the bactericidal activity of EGCG, but can be overcome with higher concentrations of EGCG that saturate the inhibitory components in serum.

Green tea and EGCG inhibit the growth of *B. anthracis* Ames. The *B. anthracis* capsule is a virulence factor that acts as a protective coat surrounding the bacilli preventing phagocytosis and preventing molecules from reaching the cell wall of the bacilli (1). Thus, the activity of EGCG against the encapsulated *B. anthracis* Ames was examined. First, the growth of virulent bacilli was examined in BHI +/- FBS with 70 µM or 1.6mM EGCG. Notably, even in the presence of FBS, which was shown earlier to interfere with killing activity of green tea, 1.6 mM EGCG resulted in a three log decrease in CFU ml^{-1} over the course of 6 h (Fig. 4a & b). For cultures treated with 70 µM EGCG, although no CFU count differences were seen, smaller colonies resulted when grown compared to time-matched cultures that received no EGCG. (Fig. 4c). These data suggest that strain differences (ANR versus Ames) contribute to differential susceptibilities to EGCG. EGCG was also evaluated against the growth of *B. anthracis* Ames in human blood. At 24 h, there was a significant one log reduction in CFU ml^{-1} in the presence of EGCG compared to cultures without. As noted before, the lower potency of 1.6 mM EGCG in

blood (as compared to growth media) might be attributed to differences in the amount of inhibitory components in human versus bovine sera.

DISCUSSION

Previous work has showed that black tea is bactericidal towards the unencapsulated, avirulent Sterne strain of *B. anthracis* (28). Our work demonstrates the ability of green tea and its constituent catechin EGCG to inhibit the growth of and kill the unencapsulated *B. anthracis* ANR strain. Notably, our investigation also shows that green tea and EGCG exhibit bactericidal activity against an encapsulated strain, *B. anthracis* Ames.

Mechanistic studies of EGCG's bactericidal activity against the Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) have revealed that EGCG binds to peptidoglycan, resulting in impaired cell division and cell lysis (6). Thus, EGCG likely binds to the cell wall of *B. anthracis* to cause lysis. Moreover, EGCG's activity against encapsulated *B. anthracis* suggests that the capsule is permeable to EGCG or there may be areas devoid of capsule, allowing the compound to reach the peptidoglycan beneath the capsule. Thus, in addition to inhibiting the *B. anthracis* LT (17), EGCG is also bactericidal toward *B. anthracis.*

The highest concentration of EGCG measured in human plasma is 7 µM after ingestion of a single 1600 mg dose. The large difference in amount of EGCG consumed and the concentration found in systemic circulation is due to the breakdown of EGCG by intestinal micro-organisms, poor absorption by the small intestines, and its active removal by efflux transporters (29-31). Although we did not see effective killing at 7 µM, it may be possible to achieve higher concentrations in blood if EGCG is administered intravenously. EGCG has been shown to have minimal toxicity in animals and humans (32). Indeed, injection of EGCG to

achieve an estimated circulating dose of 100 μM in Fischer 344 rats allowed some to recover from LT-induced toxicity. Moreover, rats and mice that were injected with EGCG alone did not experience adverse reactions (17). Thus, intravenous administration of higher and repeated dosing of EGCG should be evaluated for safety and efficacy in animal models of anthrax.

To increase the bioavailability of EGCG in the systemic circulation, alternative delivery systems or chemical modification of EGCG should also be considered. A recent report has shown that the nanoencapsulation of EGCG allows sustained release and site specific targeting of the molecule (33). In addition, peracetylated EGCG has increased biological potency and bioavailability, though once converted back to the parent compound, it undergoes the same degradation and metabolism described previously (31). Alternatively, a different route of administration that prevents EGCG from being rapidly metabolized or inactivated, such as a nebulized form of EGCG, should be considered. Others have shown that the nebulized form of tea catechins are safe and effective against methicillin resistant *Staphylococcus aureus* in the respiratory tract of humans (34). Therefore, future studies should address alternative routes of delivery for EGCG in order to avoid metabolic processes, chemical degradation and inactivation by serum albumin and other blood constituents.

Further, it is important to explore the pharmacodynamic relationship of EGCG with existing antibiotics. Importantly, synergy with another anti-microbial would reduce the required amount of EGCG to achieve a bactericidal concentration *in vivo*. Zhao and colleagues have shown synergy between EGCG and β-lactams (6). Although some strains of *B. anthracis* have been reported as resistant, most strains are susceptible to β-lactams (35). Thus, EGCG might synergize with β-lactams to enhance killing of *B. anthracis*.

Although EGCG has bactericidal activity against *Bacillus anthracis,* shown here in our study, and against other pathogens (6, 36), it is important to note that other major catechins, found in green tea, also have a similar function (36). Less abundant than EGCG, catechins such as epigallocatechin and epicatechin gallate are also antibacterial agents. Moreover, the bactericidal activity of green tea is likely the sum, if not from the synergistic combination, of the individual catechins (36, 37). Thus, future work should explore the anti-microbial activity of other green tea catechins individually and in combination with each other and EGCG. Finally, as EGCG remains a promising drug candidate against *B. anthracis,* given its anti-bacterial and anti-LT activity, and along with other pathogens, more efforts should be directed towards overcoming some of EGCG's pharmacokinetic challenges.

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DISCLAIMER

The opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. Research involving human subjects adhered to the principles identified in the Belmont Report (1979) and, unless certified as exempt, was conducted in accordance with an IRB-approved protocol and in compliance with Department of Defense, Federal, and State statutes and regulations relating to the protection of human subjects.

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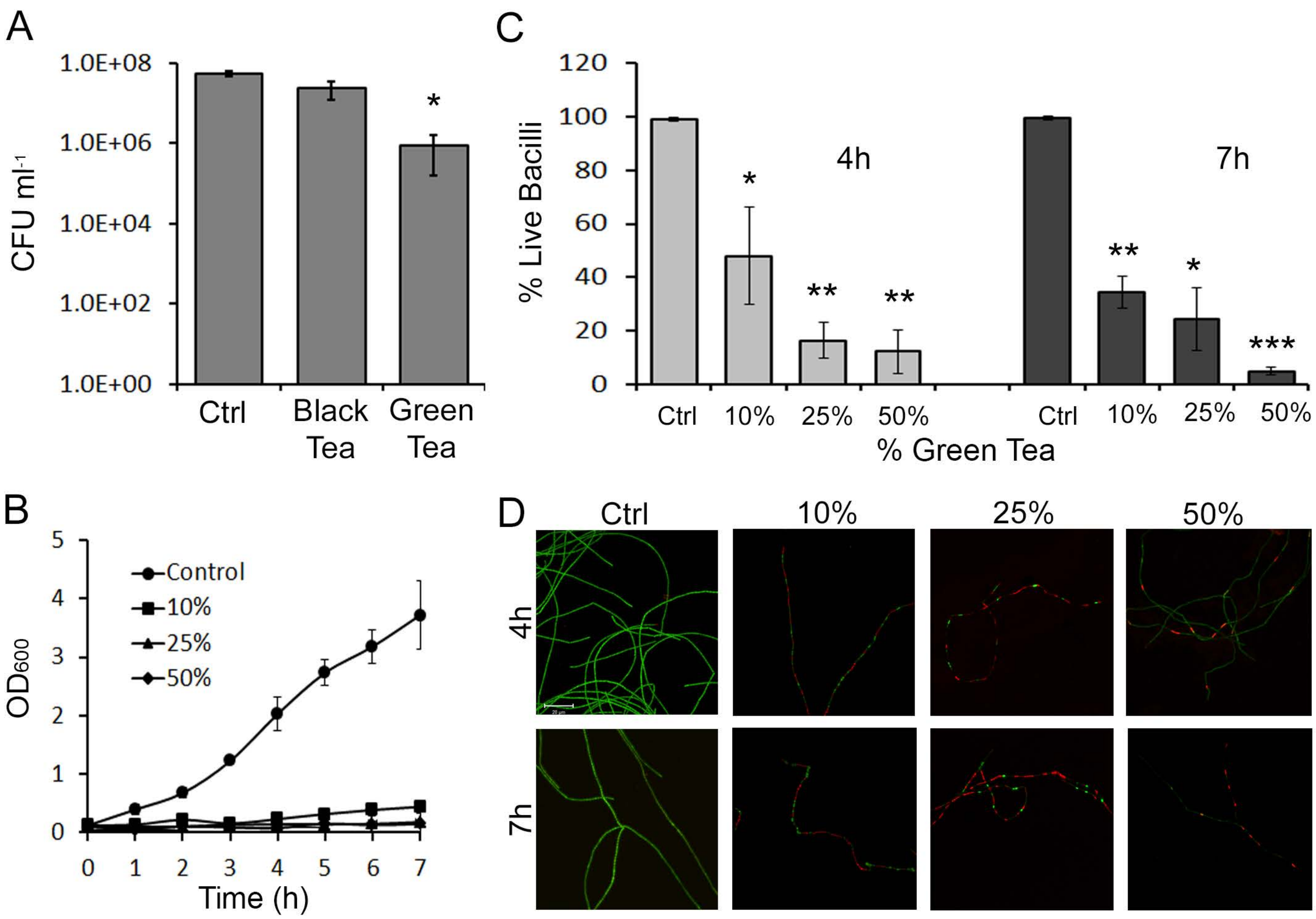
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Figure 1

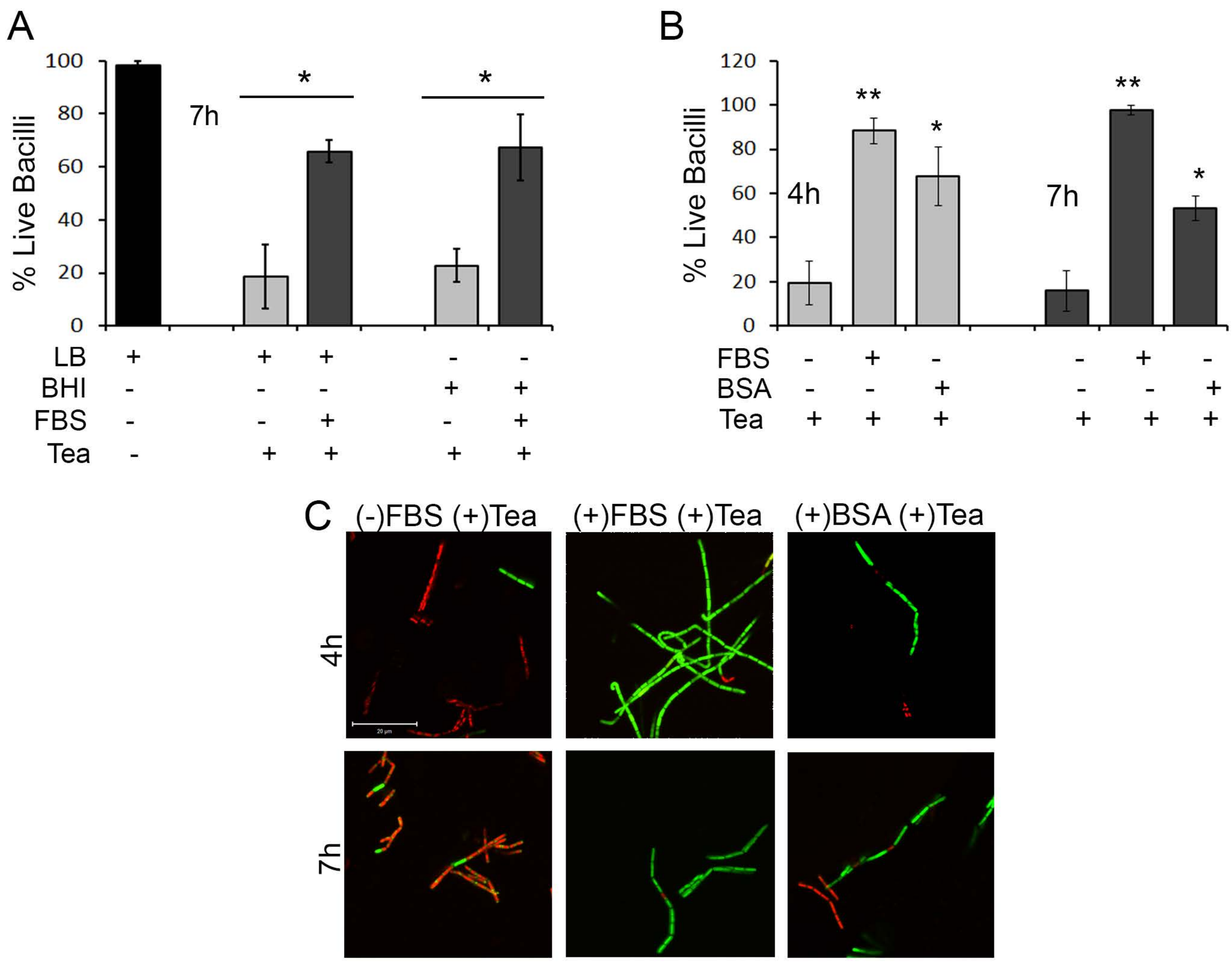
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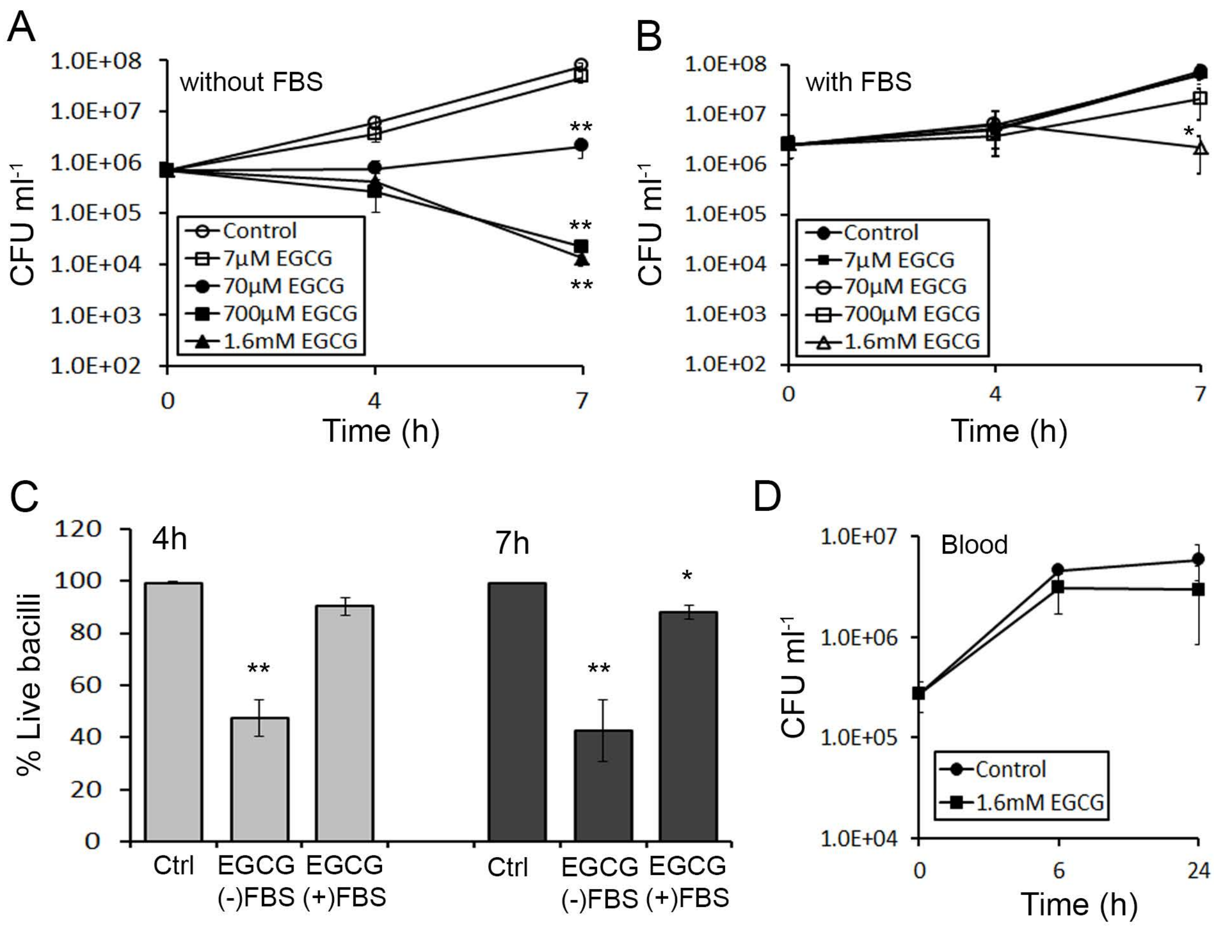
Figure 2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CFU ml-1
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700μM EGCG
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1.0E+04
CFU ml-1
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1.6mM EGCG
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Time (h)

Figure 3

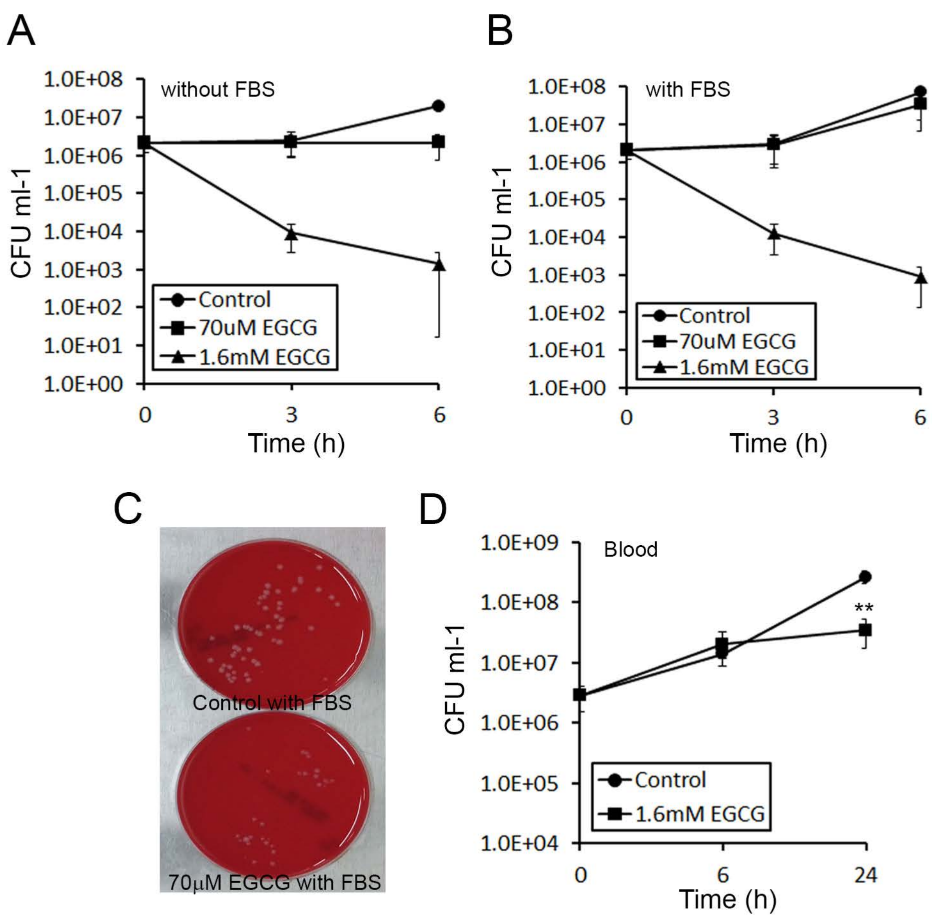
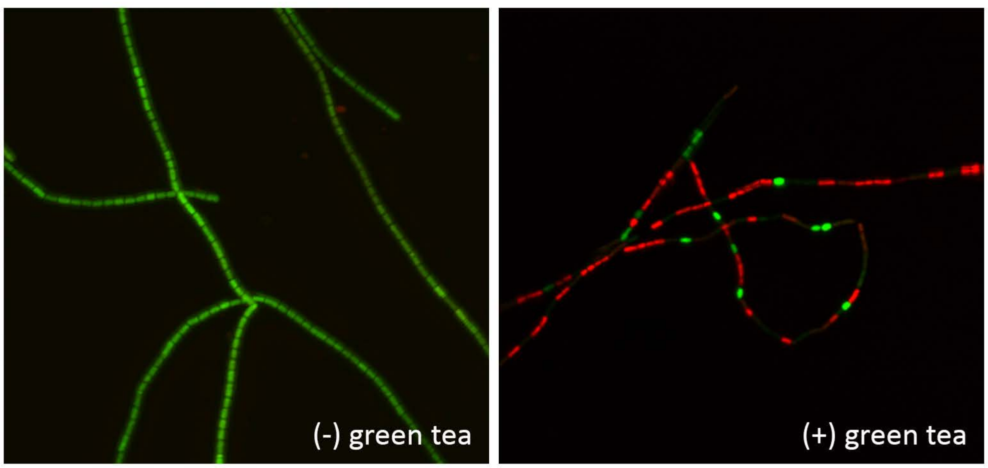

A
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU ml-1
without FBS
Control
70uM EGCG
1.6mM EGCG
0
3
6
Time (h)
B
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU ml-1
with FBS
Control
70uM EGCG
1.6mM EGCG
0
3
6
Time (h)
C
Control with FBS
70μM EGCG with FBS
D
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
CFU ml-1
Blood
**
Control
1.6mM EGCG
0
6
24
Time (h)

Figure 4



Graphical Abstract